Solid phase synthesis of O-glycoopioid peptides related to dermorphin

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Glycosylation of Fmoc-hydroxyamino acids with β-D-glucose pentaacetate has been carried out in the presence of several Lewis acids and BF$_3$.Et$_2$O has been found to be the most suitable one. Thus, 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl derivatives of Fmoc-Ser/Thr/Tyr are prepared in a single step in reasonably good yields and high purity. The O-glycosylated derivatives are then converted to their corresponding trichlorophenyl esters for use in the solid phase synthesis of five glycoopioid peptides related to dermorphin. The effect of glycosylation on biological activity of dermorphin has been studied. Among the peptides synthesized, [Tyr(β-D-Glc)^5]dermorphin and [Ser(β-D-Glc)^5, Tyr^7)]dermorphin exhibit considerable analgesic activity of about 80-90% compared to morphine and antidiarrhoeal activity of about 50% compared to dermorphin.

The growing realisation of the biological importance of glycopeptides and glycoproteins has focussed considerable attention on their chemical synthesis[1-3]. As direct glycosylation of peptides is unsatisfactory, suitably protected glycosylated amino acids are preferred in glycopeptide synthesis. For this, the protecting groups have to be judiciously chosen as the glycosidic bonds are sensitive to strong acids and bases. For the synthesis of O-glycopeptides, O-glycosylated N^α-9-fluorenylmethoxycarbonyl (Fmoc) amino acids are presently used. Several approaches for their preparation and their subsequent use in the chemical synthesis of O-linked glycopeptides have been reported[4-7]. For the preparation of O-glycosylated Fmoc-amino acids, crude 3-O-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)amino acids were first obtained by catalytic hydrogenation of the corresponding N^α-benzyloxycarbonyl-O-glycosylated amino acid benzyl esters followed by reaction with Fmoc-succinimidyl carbonate [4,5]. They have also been prepared by cleavage of acid labile esters of the N^α-Fmoc protected O-glycosylated amino acids[6]. Such routes, however, are tedious and lengthy. This paper describes a simpler procedure where O-glycosylation of Fmoc-hydroxyamino acids is effected in presence of BF$_3$.Et$_2$O without protection of their carboxyl group. These have subsequently been converted to the corresponding 2,4,5-trichlorophenyl esters for use in the solid

phase synthesis of the glycoopioid peptides related to dermorphin.

Dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH$_2$), a morphinomimetic heptapeptide isolated from the skin of the South American frog *Phyllomedusa sauvegei* exhibits high opioid agonist activity[8-10]. Several studies on the synthesis, conformation and biological activity of dermorphin and its analogues have been reported. However, there are no reports as yet on glycosylation of dermorphin and its effect on biological activity. In the present studies, five analogues of dermorphin with modifications at positions 5 and/or 7 and glycosylation at position 1 or 5 were synthesized. In one analogue, Tyr^5 was replaced by Tyr(β-D-Glc)^5 while in the other Tyr^1 was replaced by Tyr(β-D-Glc)^1 residue. As [Tyr^7]dermorphin has been reported to possess almost twice the activity of dermorphin[11], glycosylation in the [Tyr^7]dermorphin series was effected to obtain three analogues with either Tyr(β-D-Glc)^5, Ser(β-D-Glc)^5 or Tyr(β-D-Gal)^5 residue.

Different methods were tried for the preparation of O-glycosylated hydroxyamino acids, starting from N^α-Fmoc-protected derivatives. During this step, the carboxyl group was not protected and glycosylation reaction was tried with β-D-glucose pentaacetate in presence of various metal halides (Lewis acids)[12,13] like SnCl$_4$, FeCl$_3$ or BF$_3$.Et$_2$O. Glycosylation of Fmoc-Ser was also tried with

2,3,4,6-tetra-*O*-benzyl-D-glucopyranose in the presence of trifluoromethane sulfonic anhydride[14].

Among them, the reaction between Fmoc-Ser and β-D-glucose pentaacetate in presence of $BF_3.Et_2O$ was found to be the most efficient and the resultant *O*-glycosylated derivative was obtained in better yield and the purity was also satisfactory as determined by HPLC. The reaction appears to proceed through the formation of a stabilized cyclic acyloxonium intermediate which upon activation by a Lewis acid will allow nucleophilic attack by a glycosyl acceptor to afford anometrically pure β-glycoside[15] (Scheme I). The

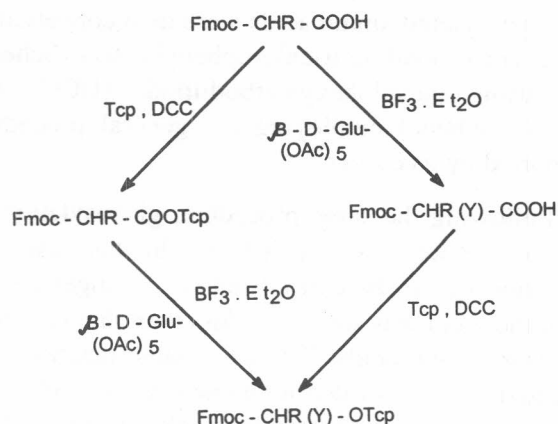(i) $BF_3 . Et_2O$, CH_3CN ;   (ii) Fmoc - Ser or Fmoc - Thr
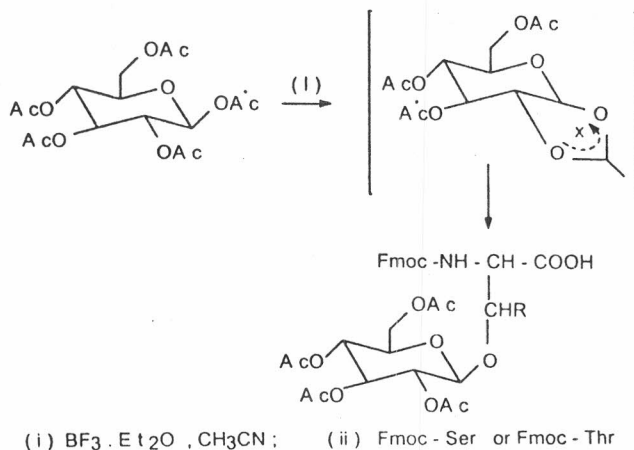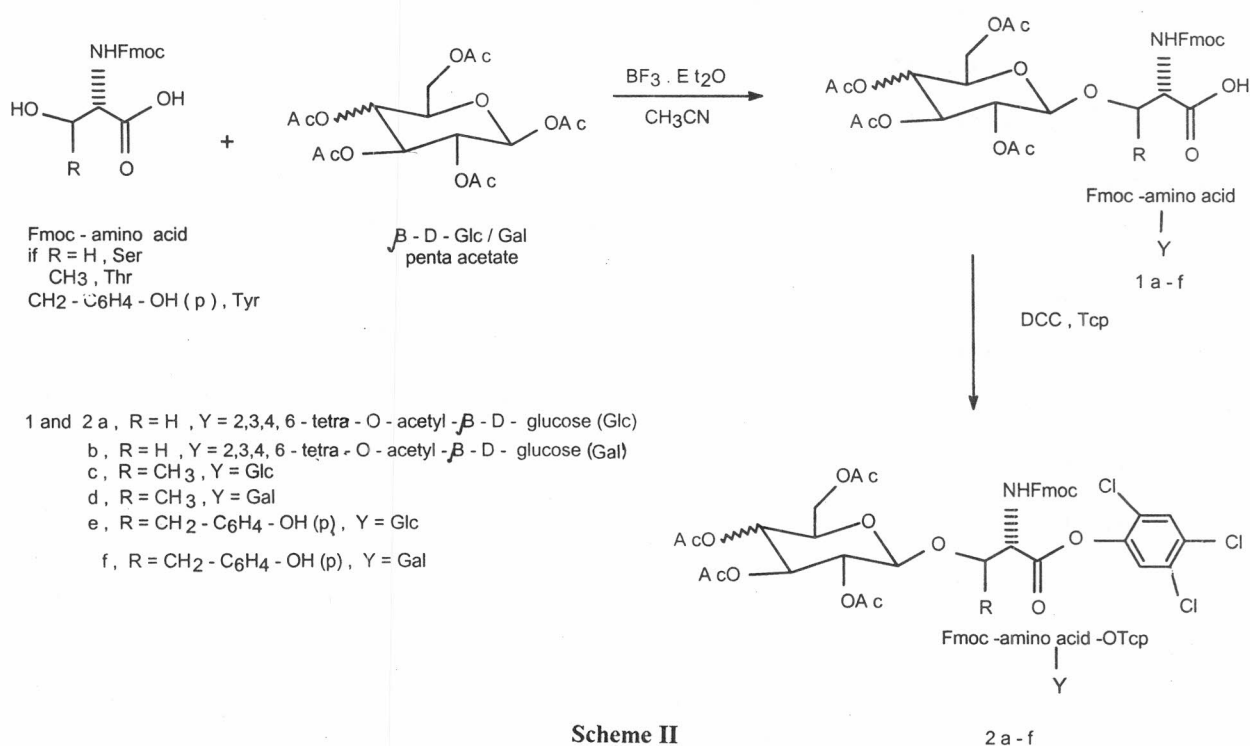
Scheme I

O-glycosylated amino acids were then converted to their corresponding trichlorophenyl esters (Scheme II) using dicyclohexylcarbodiimide (DCC) and trichlorophenol employing the general procedure reported by us earlier[16].

Following the same procedure, glycosylation of Fmoc-Tyr was also carried out. In this case, the reaction had to be carried out for a longer period and the yield was somewhat less than that for other hydroxyamino acids. When the same reaction was carried out at elevated temperatures (40°, 60° and 80°), no improvement in the yield of the product was observed. The yield and physical constants of the glycosylated amino acid derivatives are listed in Table I. All the glycosylated amino acids are fine crystals instead of the foamy solids generally reported by others.

Alternately, Fmoc-Ser was converted to the active trichlorophenyl ester and then glycosylation was effected (i) with β-D-glucose pentaacetate in the presence of $BF_3.Et_2O$ (Scheme III), (ii) using 2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide with $Hg(CN)_2$ and nitromethane in benzene at 80° by employing the procedure followed for the preparation of Fmoc-Hyp[β-D-Glc(OAc)$_4$]-OMe[14]. The reactions were very slow and the yields were

Fmoc - amino acid
if R = H , Ser
   CH_3 , Thr
   $CH_2 - C_6H_4 - OH (p)$, Tyr

β - D - Glc / Gal
penta acetate

Fmoc -amino acid

1 a - f

DCC , Tcp

Fmoc -amino acid -OTcp

Y

1 and 2 a , R = H , Y = 2,3,4, 6 - tetra - O - acetyl -β - D - glucose (Glc)
       b , R = H , Y = 2,3,4, 6 - tetra - O - acetyl -β - D - glucose (Gal)
       c , R = CH_3 , Y = Glc
       d , R = CH_3 , Y = Gal
       e , R = $CH_2 - C_6H_4 - OH (p)$, Y = Glc
       f , R = $CH_2 - C_6H_4 - OH (p)$, Y = Gal

Scheme II   2 a - f

Fmoc - CHR - COOH

Tcp , DCC   BF$_3$. E t$_2$O

β - D - Glu-
(OAc)$_5$

Fmoc - CHR - COOTcp   Fmoc - CHR (Y) - COOH

BF$_3$. E t$_2$O   Tcp , DCC

β - D - Glu-
(OAc)$_5$

Fmoc - CHR (Y) - OTcp

R = CH_2OH or $CH(CH_3)OH$ or $CH_2 C_6H_4OH$ (p)
Y = 2 , 3 , 4 , 6 - tetra - O - acetyl - β - D - glucose / galactose
Tcp = 2 , 4 , 5 - trichlorophenol

Scheme III

also low. Thus, glycosylation of Fmoc-hydroxyamino acid with β-D-glucose pentaacetate in the presence of $BF_3.Et_2O$ was found to be efficient when the α-carboxyl group is free.

The utility of these Fmoc-O-glycosylated amino acid derivatives in solid phase peptide synthesis has been demonstrated by the synthesis of five analogues of dermorphin having O-glycosylated serine or tyrosine at positions 1 or 5. The synthesis of the protected heptapeptides on a conventional Merrifield resin (chloromethylated polystyrene crosslinked with 2% divinylbenzene) was accomplished by using appropriate Fmoc-amino acid trichlorophenyl esters in presence of 1-hydroxybenzotriazole (HOBt)[11] with suitable modifications. For the cleavage of peptide from the resin, direct ammonolysis (methanolic ammonia at 0° for 72 h) was not found to be appropriate because of the characteristic base lability of the O-glycosidic bonds. Hence, the protected glycopeptide amides have been obtained in a two step process. The protected peptide was cleaved from the solid support by KCN/methanol (MeOH) method[17] to get the free acid which was then

Table I—Analytical data of Fmoc-O-glycosylated amino acids and their trichlorophenyl esters

Sl. No.	Compound	Yield (%)	m.p. (^{O}C)	$[\alpha]_D^{25}$	R_f values	
					R_f A	R_f B
1	Fmoc-Ser[Glc(OAc)$_4$]*	53	86-88	+20.0° (c1, CHCl$_3$)	0.49	0.62
2	Fmoc-Ser[Gal(OAc)$_4$]	52	foamy solid	+ 16.8° (c l.l, CHCl$_3$)	0.50	0.65
3	Fmoc-Thr[Glc(OAc)$_4$]**	50	120-121	− 17.1° (c 1, MeOH)	0.56	0.70
4	Fmoc-Thr[Gal(OAc)$_4$]#	51	83-85	− 4.6° (c 1.12, MeOH)	0.45	0.72
5	Fmoc-Tyr[Glc(OAc)$_4$]	43	108-110	+ 20.0° (c 0.5 CHCl$_3$)	0.73	0.88
6	Fmoc-Tyr[Gal(OAc)$_4$]	40	93-95	+ 50.0° (c l, CHCl$_3$)	0.52	0.71
7	Fmoc-Ser[Glc(OAc)$_4$]-OTcp	39	foamy solid	+13.7° (c 1,CHCl$_3$)	0.43	0.73
8	Fmoc-Ser[Gal(OAc)$_4$]-OTcp	62	96-98	+ 6.3° (c 0.8, CHCl$_3$)	0.78	0.86
9	Fmoc-Thr[Glc(OAc)$_4$]-OTcp	60	146-148	− 58.7° (c 0.9, CHCl$_3$)	0.86	0.95
10	Fmoc-Thr[Gal(OAc)$_4$]-OTcp	60	128-130	− 55.5° (c 0.9, CHCl$_3$)	0.88	0.91
11	Fmoc-Tyr[Glc(OAc)$_4$]-OTcp	56	105-107	+ 16.6° (c 1.2, CHCl$_3$)	0.90	0.84
12	Fmoc-Tyr[Gal(OAc)$_4$]-OTcp	55	98-101		0.89	0.84

The abbreviations used for amino acids are in accordance with recommendations of IUPAC-IUP commission on Biochemical Nomenclature published in "Pure and Applied Chemistry", 40(1974) 314. Elemental analysis of these compounds were found to be satisfactory. * Ia, See Ref.7, reported : foam ; $[\alpha]_D$ +25° (c 0.4, CHCl$_3$) ; ** Ic, See Ref. 4, reported : m.p. 126° dec ; $[a]_D$ - 17.1° (c 0.61, MeOH) ; # Id, See Ref. 4, reported : m.p. 80-82° dec ; $[a]_D$ - 4.6° (c l.12, MeOH).

converted to an amide by using DCC/HOBt/aqueous ammonia[18].

Biological activities

The opiate agonistic properties of the synthetic *O*-glycopeptides were studied using the co-axially, electrically stimulated contractions of the isolated guinea pig ileum (GPI) *in vitro*[19] and *in vivo* analgesic activity using Eddy's hot plate method[20]. The antidiarrhoeal activity (*in vivo*) was determined by the charcoal meal test based on the inhibition of the gastrointestinal motility[21]. The activities were determined relative to morphine sulphate as standard and the results are summarised in Table II.

In the GPI assay, the synthetic analogues, except [Tyr(Glc)^1]dermorphin, exhibited 1/10th the activity of morphine; [Tyr(Glc)^1]dermorphin being almost inactive. In the analgesic assay, all the glycosylated peptides showed considerable activity. Glycosylation of Tyr at position 5 in dermorphin and substitution of Tyr^5 by *O*-glycosylated serine in [Tyr^7]dermorphin led to peptides **6** and **7** with considerable analgesic activity, being respectively 0.8823 and 0.8240 times as active as morphine. In [Tyr^7]dermorphin, glycosylation of Tyr^5 by either β-D-glucopyranose or β-D-galactopyranose unit gave peptides **8** and **9** which showed about 70% of the analgesic activity compared to morphine. These two peptides, though less active, are equipotent in all the assays, indicating that substitution of glucopyranose for galactopyranose residue, in the dermorphin series does not have any influence on activity. Peptides **6**

and **7** exhibited considerable antidiarrhoeal activity compared to both morphine and dermorphin while the other peptides (peptides **8**, **9** and **10**) were almost inactive in this assay. Thus, peptides **8**, **9** and **10** are unique in possessing only analgesic activity to the virtual exclusion of the other two activities. From the biological activities listed in Table II, it is clear that the glycopeptides have much less GPI activity as compared to morphine or the parent compound. While the analgesic activities are comparable or less than that of morphine, it is much less active than dermorphin. The antidiarrhoeal activities are also less as compared to dermorphin. Also, there is a certain degree of selectivity in the activities and such peptides with high degree of specificity may prove useful in biological applications.

Experimental Section

All the amino acids used, except glycine, are of L-configuration unless otherwise specified. Melting points were determined using Leitz-Wetzlar melting point apparatus and are uncorrected. Thin layer chromatography (TLC) was performed on precoated silica gel G plates by ascending method using the following solvent systems (A) chloroform-methanol-acetic acid (40:2:1, v/v/v), (B) ethyl acetate-hexane-acetic acid (80:17:3, v/v/v), (C) *n*-butanol-acetic acid-water (4:1:1, v/v/v, upper phase) and (D) *n*-butanol-acetic acid-water (4:1:5, v/v/v, upper phase) and the R$_f$ values are designated as R$_f$A, R$_f$ B, R$_f$ C and R$_f$ D respectively. Analytical HPLC was performed on Waters LC 3000 system equipped with Waters

Table II—Biological activity of synthetic *O*-glycopeptides related to dermorphin

Peptide	GPI		Analgesic	Antidiarrhoeal *
	Mean IC$_{50}$ nM	Relative Potency		
Morphine sulphate	—	1.0000	1.0000	1.0000
[Tyr(β-D-Glc)^5]dermorphin **6**	255.64	0.1495 ± 0.019	0.8823	0.443
[Ser(β-D-Glc)^5, Tyr^7]dermorphin **7**	322.06	0.1417 ± 0.03	0.8240	0.513
[Tyr(β-D-Glc)^5, Tyr^7]dermorphin **8**	214.07	0.1101 ± 0.016	0.7294	0.348
[Tyr(β-D-Gal)^5, Tyr^7]dermorphin **9**	317.47	0.116 ± 0.021	0.6941	0.116
[Tyr(β-D-Glc)^1]dermorphin **10**	613.35	0.058 ± 0.01	0.5392	0.197
Dermorphin[11]	0.1567±3.3×10^{-3}	42.57	10.15	0.9367
[Try^7]dermorphin[11]	0.083	78.71	14.38	0.9984

*Percentage inhibition of charcoal meal transit relative to morphine (= 35 ± 3).

484 tunable absorbance UV detector and a Millipore 745 data module. Waters RP C_{18}-deltapak column (3.9 mm × 30 cm, 15 µ, spherical) was used for analysis. Optical rotations were recorded using automatic digital AA-10 polarimeter (Optical Activity, UK). Amino acid analyses were carried out using Waters PICO-TAG system. For elemental analysis, amino acid derivatives dried over P_2O_5 under vacuum for 24 h were used. PMR spectra were determined on Brucker ACF instrument. The coupling and deprotection reactions, during the course of the synthesis, were monitored by Kaiser's ninhydrin test[22]. Fmoc-amino acids and their 2,4,5-trichlorophenyl esters were prepared by following the reported procedures[23]. However, Fmoc-Ser/Thr-OTcp whose preparation was reported to be difficult[24], have now been prepared in a crystalline form and employed during the present investigations.

Fmoc-Ser/Thr-OTcp: Fmoc-Ser/Thr (10 mmole) and 2, 4, 5-trichlorophenol (10 mmole) were dissolved in dry ethyl acetate (25 mL) and the solution was cooled to 0°. Then DCC (12 mmole) was added and the mixture was stirred for 4 h at 0° and overnight at room temperature. The mixture was cooled to - 10° and insoluble dicyclohexylurea (DCU) was filtered off. The filtrate was washed with H_2O, 5% $NaHCO_3$ solution, H_2O and saturated NaCl solution. It was then dried over Na_2SO_4 and concentrated *in vacuo*. The residue was recrystallised from ethyl acetate-hexane. Fmoc-Ser-OTcp: Yield 68%; m.p. 118-20°; R_fA 0.53; $[\alpha]_D^{25}$ −20.3° (c 1, $CHCl_3$) (Calc. for $C_{24}H_{18}NO_5Cl_3$: C, 56.86; H, 3.55; N, 2.76. Found: C, 56.91; H, 3.59 N, 2.80); HPLC: t_R 18.51 min (elution: isocratic 35% aq. acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min and UV monitoring at 220 nm); ^{1}H NMR (400 MHz, $CDCl_3$): 4.00-4.15 (m, 2H, Ser β-H_2), 4.26 (t, 1H, Fmoc-CH), 4.43 (d, 2H, Fmoc-CH_2), 5.66 (bd, 1H, NH), 7.23-7.88 (m, 10H, ArH). Fmoc-Thr-OTcp: Yield 65%; m.p. 106-108°; R_fA 0.58; $[\alpha]_D^{25}$ −26.8° (c 1, $CHCl_3$) (Calc. for $C_{25}H_{20}NO_5Cl_3$: C, 57.63; H, 3.94; N, 2.69. Found: C, 57.68; H, 3.96; N, 2.71); HPLC: t_R 17.53 min (elution: isocratic 35% aq. acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min and UV monitoring at 220 nm); ^{1}H NMR (400 MHz, $CDCl_3$): 1.45 (d, 3H, Thr γ-H), 4.25 (m, 1H, Fmoc-CH), 4.43 (dq, 1H, Thr β-H), 4.56 (d, 2H, Fmoc-CH_2), 4.75 (dd, 1H, Thr α-H), 5.80 (d, 1H, Thr-NH), 7.30-7.81 (m, 10H, ArH).

Fmoc-O-(tetra-O-acetyl-β-D-glucopyranosyl)-Ser 1a. To a solution of Fmoc-Ser (2.71 g, 8.28 mmole) and β-D-glucose pentaacetate (3.32 g, 8.5 mmole) in dry acetonitrile (10 mL) at room temperature was added boron trifluoroetherate (2.61 mL, 20.7 mmole). After 2-3 h (reaction monitored by TLC), the reaction mixture was diluted with CH_2Cl_2 (30 mL) and washed with $1M$ HCl (2×10 mL). The aq. layer was then extracted with CH_2Cl_2 (2×10 mL). The organic layers were pooled, washed with water, dried over anhyd. Na_2SO_4 and evaporated. The gum obtained was subjected to column chromatography over silica gel using ethyl acetate-hexane-acetic acid (18:17:3, v/v/v) as eluant. The fractions corresponding to the product were pooled, concentrated and recrystallised from CH_2Cl_2-hexane. The final purity was established by analytical HPLC (Figure 1a). ^{1}H NMR (300 MHz, acetone-d_6): 1.97, 1.99, 2.01 and 2.03 (4s, 3H each, Ac), 3.79 (dd, 1H, H-β), 4.07 (dd, 1H, H-6), 4.39 (m, 1H, H-α), 4.89 (d, 1H, H-1), 4.93 (dd, 1H, H-2), 5.03 (t, 1H, H-4), 5.28 (t, 1H, H-3), 6.54 (d, 1H, NH), 7.20 - 7.74 (m, 8H, ArH).

By following the same procedure, Fmoc-O-(tetra-O-acetyl-β-D-galactopyranosyl)Ser **1b** and Fmoc-O-tetra-O- acetyl-β-D-gluco/galactopyranosyl)Thr **1c/1d** were prepared. The yields and physical data are listed in Table I.

Fmoc-O-(tetra-O-acetyl-β-D-glucopyranosyl)-Tyr 1e. To a mixture of Fmoc-Tyr (2.01 g, 5 mmole) and β-D-glucose pentaacetate (3.9 g, 10 mmole) in dry acetonitrile (6 mL) was added boron trifluoroetherate (1.62 mL, 12.5 mmole) and stirred at room temperature overnight. The reaction mixture was then diluted with CH_2Cl_2 (30 mL) and washed with water (3 × 10 mL). The organic layer was dried over anhyd. Na_2SO_4 and concentrated *in vacuo*. The gum obtained was purified by flash chromatography over silica gel G using ethyl acetate - hexane (6:4, v/v) as eluant and the product was recrystallised from ethyl acetate-hexane. The purity of the compound was checked by analytical HPLC (Figure 1b). ^{1}H NMR (300 MHz, acetone-d_6) 1.97, 1.99, 2.00 and 2.02 (4s, 3H each, Ac), 2.98 (dd, 1H, H-β), 3.23 (dd, 1H, H-β), 4.49 (m, 1 H, H-α), 5.08 (t, 1H, H-4), 5.14 (dd, 1H,
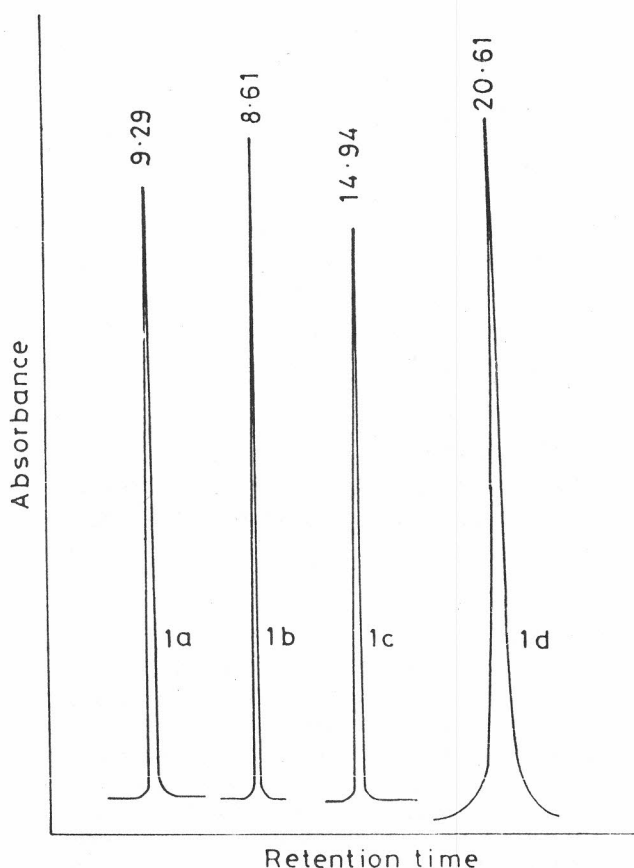
Figure 1a—Elution profile of pure Fmoc-*O*-(tetra-*O*-acetyl-β-D-glucopyranosyl)Ser. Elution: isocratic 35% aq. acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min and UV monitoring at 220 nm; (b) Elution profile of pure Fmoc-*O*-(tetra-*O*-acetyl-β-D-glucopyranosyl)Tyr. Elution: isocratic 35% aq. acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min and UV monitoring at 220 nm; (c) Elution profile of pure Fmoc-*O*-(tetra-*O*-acetyl-β-D-glucopyranosyl)Tyr-OTcp. Elution: isocratic 35% aq. acetonitrile containing 0.1% TFA at a flow rate of 1 mL/min and UV monitoring at 220 nm; (d) Analytical RP-HPLC profile of [Tyr(β-D-Glc)^5]dermorphin. Eluant A, aq. 0.1% TFA; eluant B, 0.1% TFA in acetonitrile. Flow rate 1 mL/min; UV monitoring at 220 nm. Elution: linear gradient of 25-100% B over 30 min.

H-2), 5.24 (d, 1H, H-1), 5.34 (t, 1H, H-3), 6.74 (d, 1H, NH), 6.98 - 7.28 (2d, 4H, Tyr ε, δ-H).

Fmoc-*O*-(tetra-*O*-acetyl-β-D-Glc/Gal)Ser/-Thr/Tyr-OTcp (2a-f): To a well stirred solution of Fmoc-*O*-(tetra-*O*-acetyl-β-D-Glc/Gal)Ser/Thr/-Tyr (10 mmole) and 2, 4, 5-trichlorophenol (11 mmole) in dry ethyl acetate (35 mL) cooled in an ice bath was added DCC (11 mmole) and stirring continued for 2 h and at room temperature for 2 h. The separated DCU was filtered off and the filtrate concentrated *in vacuo*. The product was recry-

stallised from ethyl acetate-hexane. The yields and physical constants are listed in Table I. The purity of the compounds were found to be >98% by HPLC (Figure 1c).

Peptide synthesis

Synthesis of [Tyr (β-D-Glc)^5]dermorphin

Boc-Ser-OCH$_2$-C$_6$H$_4$-resin. A mixture of Boc-Ser (5 mmole), Merrifield resin (3.0 g), absolute ethanol (12 mL) and NEt$_3$ (6 mmole) was stirred at 90° for 24 hr under anhyd. conditions. The esterified resin was collected and washed successively with ethanol, water, ethanol, CH$_2$Cl$_2$ and methanol and dried over P$_2$O$_5$ *in vacuo,* yield 3.42 g. The Ser content on the resin was estimated to be 0.39 mmole/g by the Volhard method[25].

Boc-Tyr-D-Ala-Phe-Gly-Tyr[β-D-Glc(OAc)$_4$]-Pro-Ser-OCH$_2$-C$_6$H$_4$-resin 3. Boc-Ser-OCH$_2$-C$_6$H$_4$-resin (1.7 g) was taken in a solid phase vessel and synthesis continued manually using appropriate Fmoc-amino acid trichlorophenyl esters in the presence of 1-hydroxybenzotriazole as reported earlier[11] with suitable modifications. Cleavage of Fmoc group was effected by using 60% diethylamine in dimethylformamide (DMF). However, after the coupling of glycosylated amino acid residue, cleavage of Fmoc group was accomplished with 50% piperidine in DMF.

The chain elongation was then carried out by stepwise addition of Fmoc-Pro-OTcp (1.06 g, 3 equiv.), Fmoc-Tyr[β-D-Glc(OAc)$_4$]OTcp (1.85 g, 3 equiv.), Fmoc-Gly-OTcp (0.98 g, 3 equiv.), Fmoc-Phe-OTcp (1.16 g, 3 equiv.), Fmoc-D-Ala-OTcp (1.00 g, 3 equiv.) and Boc-Tyr-OTcp (0.95 g, 3 equiv.) in the presence of HOBt (0.089 g, 1 equiv.) in DMF. The reaction time for coupling of each amino acid was generally 60-120 min except for glycosylated amino acid which took 160-180 min. After each coupling, the resin was washed with DMF (3 × 2 min), isopropanol (3×2 min), CH$_2$Cl$_2$ (3×2 min) and ether (3×2 min). After the completion of the synthesis, the protected peptide resin was dried *in vacuo* over P$_2$O$_5$ and KOH. Yield, 2.32 g.

Boc-Tyr-D-Ala- Phe-Gly-Tyr(β-D-Glc)-Pro-Ser 4. The protected peptide resin (3, 2.0 g) was stirred with KCN (200 mg/g) and 95% aq. methanol (30 mL/g) at room temperature for 24 hr[17]. The solution was neutralized with 1*N* HCl. Resin was filtered and washed with hot methanol. The combined filtrate and washings were

concentrated to dryness *in vacuo.* To this was added 8-10 mL of distilled water and brought the *p*H to 2 using 1*N* HCl. White precipitate separates out which was extracted with ethyl acetate three times. The organic layers were pooled, washed with water and dried over Na_2SO_4. The protected peptide obtained, after concentration *in vacuo,* was crystallised from methanol-ether, yield, 0.342 g (68% based on the extent of Ser esterified to the resin).

Boc-Tyr-D-Ala-Phe-Gly-Tyr(β-D-Glc)-Pro-Ser-NH$_2$ 5. The protected peptide (**4**, 0.212 g, 0.2 mmole) was dissolved in DMF (1.0 mL) and HOBt (0.044 g, 0.33 mmole) and DCC (0.04 g, 0.21 mmole) were added[18]. The mixture was stirred for 30 min. Then 25% aq. ammonia (0.05 mL, 0.65 mmole) was added at 0^0, and stirring continued for 2 hr at 0° and overnight at room temperature (reaction monitored by TLC). The insoluble by-products were filtered off and the filtrate was diluted with CH_2Cl_2 (10 mL). This solution was washed with 2% $NaHCO_3$ solution (3 × 5 mL), water (5 mL), 5% citric acid (2×3 mL) and water (5 mL), dried over anhyd. Na_2SO_4 and evaporated *in vacuo* to give the protected peptide amide which was recrystallised from methanol-ether (1:3), yield, 0.16 g (73%).

Try-D-Ala-Phe-Gly-Tyr(β-D-Glc)-Pro-Ser-NH$_2$ 6. The protected peptide amide (**5**, 0.098 g, 1 mmole) was treated with TFA: CH_2Cl_2 (1:1) for 1 hr (reaction monitored by TLC). After complete deprotection of Boc group, the reaction mixture was concentrated *in vacuo* and concentration repeated twice by addition of MeOH. The resulting gum was then dissolved in ethyl acetate and neutralized with 2% $NaHCO_3$ solution to get the free peptide amide. The crude peptide was purified on a silica gel column (30 cm × 2 cm) using ethyl acetate-hexane-acetic acid (90:10:1, v/v/v) as eluant. The purity of final peptide was checked by analytical RP-HPLC (Figure 1d), yield, 0.056 g (66%); m.p. 110-12°; R_f C 0.74; R_f D 0.65; amino acid analysis: Ser (1) 1.04, Tyr (2) 1.96, Gly (1) 0.98, Phe (1) 1.01, Ala (1) 0.97.

By following the same proforma and with appropriate modifications where necessary, the remaining four glycopeptides related to dermorphin **7** to **10** were obtained. The yields and physical data are listed in Table III.

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Table III—Analytical data of *O*-glycosylated peptides related to dermorphin *

Sl. No.	Peptide	Yield (%)	m.p. (°C)	R_f C	R_f D
1	[Tyr(β-D-Glc)^5]dermorphin **6**	66	110-112	0.74	0.65
2	[Ser(β-D-Glc)^5, Tyr^7]dermorphin **7**	63	97-99	0.75	0.69
3	[Tyr(β-D-Glc)^5, Tyr^7]dermorphin **8**	68	foamy solid	0.92	0.85
4	[Tyr(β-D-Gal)^5, Tyr^7]dermorphin **9**	68	145-148	0.86	0.78
5	[Tyr(β-D-Glc)^1]dermorphin **10**	60	106-108	0.76	0.70

* Amino acid analyses of these peptides were found to be satisfactory.

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